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ISOLATION AND CHARACTERIZATION OF MUTANTS OF *ESCHERICHIA COLI* K-12 WHICH LEAK β-GALACTOSIDASE

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SUMMARY

Five mutants of *Escherichia coli* K-12 have been isolated which release β-galactosidase into the culture medium during growth at temperatures of 23 to 42 °C. Three mutants were lactose transport negative (y^-), one mutant possesses normal transport, and another contains 15 % normal transport activity. A striking observation was that transport-negative cells were able to ferment lactose and grow on lactose as a sole carbon source. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) was rapidly split by washed transport-negative cells. The transport inhibitors thiodigalactoside (10 mM) and formaldehyde (10 mM) had little effect on this ONPG hydrolysis suggesting that β-galactosidase was external to the plasma membrane. This view was confirmed by the partial release of the enzyme by treatment of cells with lysozyme and EDTA. Evidence was presented which suggested that cell lysis was not the mechanism of protein loss from these cells. The growth medium at the end of logarithmic phase contained β-galactosidase, a small amount of glucose-6-phosphate dehydrogenase and a considerable amount of unidentified proteins which were eluted from a Sephadex G-200 column. It is concluded that the mutants possess defects in the plasma membrane–cell wall complex which allows loss of proteins from the cell. Growth on lactose by transport-negative cells is apparently due to the extracellular hydrolysis of the disaccharide followed by the uptake and metabolism of the split products.

INTRODUCTION

Three mutants of *Escherichia coli* were isolated which were unable to transport β-galactosides (y^-) but grew in lactose. Evidence is presented that this anomalous behaviour is due to the fact that in these mutants β-galactosidase 'leaks' from the intracellular compartment of the cell across the plasma membrane into the periplasmic space. The enzyme subsequently passes across the cell wall and appears in the extracellular medium. As a result of this extracellular location of the enzyme, lactose from the medium is split into glucose and galactose external to the plasma membrane and these hexoses are then taken up by the cell and utilized for growth. Two additional

Abbreviations: ONPG, *o*-nitrophenyl-β-D-galactopyranoside; TDG, D-galactopyranosyl-β-D-thiogalactopyranoside; TMG, thiomethyl-β-D-galactopyranoside; IPTG, thioisopropyl-β-D-galactopyranoside; TONPG, thio-*o*-nitrophenyl-β-D-galactopyranoside.

mutants were isolated which 'leak' β-galactosidase, one with normal membrane transport for lactose and one with a partial defect in transport. A preliminary report of this work has appeared elsewhere[1].

MATERIALS AND METHODS

Bacterial strains

The parental strain used in this study was X71 ($i^-z^+y^+a^-$, $ProC^-$, try^-, B_1^-, Sm^R, F^-) a transacetylase-negative strain derived[2] from X5072 a K-12 strain obtained from Dr Jon Beckwith. From the parental strain a series of mutants (241, 244, 415, 427 and 113B) have been isolated following mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine according to the method of Adelberg *et al.*[3]. Washed cells were incubated with the mutagen (0.1 mg/ml) for 10 min at 37 °C, diluted 100-fold into rich medium and grown overnight. The mutants were selected by the method of Müllerhill, *et al.*[4]: cells were grown in Medium 63 containing 20 mM glycerol, 0.5 mM thioisopropyl-β-D-galactopyranoside (IPTG), and 3 mM thio-*o*-nitrophenyl-β-D-galactopyranoside (TONPG) for 48 h at 37 °C from an initial inoculum of $3 \cdot 10^6$ cells. Cells were plated on McConkey plates containing 0.2 % lactose and pink or red colonies were picked for assay.

Growth of cells

The cells were grown in mineral Medium 63 (ref. 5) wich consisted of KH_2PO_4 (13.6 g), $(NH_4)_2SO_4$ (2.0 g), $MgSO_4 \cdot 7H_2O$ (0.2 g) and $FeSO_4 \cdot 7H_2O$ (5 mg) added to 1 l of distilled water and adjusted to pH 7.0 with KOH. The medium was supplemented with NaCl (0.29 %), L-tryptophan (10 μg/ml), and L-proline (100 μg/ml), and thiamine (0.5 μg/ml). Difco Bactotryptone (1 %) or, glycerol (0.4 %) served as the carbon and energy source. In order to induce alkaline phosphatase cells were grown in minimal medium buffered with 0.05 M Tris buffer (pH 7.5) containing 0.5 % glucose and 10^{-4} M sodium glycerol phosphate.

In most experiments cells were grown in 30 ml of medium in 300-ml side arm flasks. The cells were incubated by continuous shaking on a gyrotatory shaker at about 200 rev./min at 37 °C unless otherwise stipulated. Growth was initiated by transferring 0.1 ml of an 8-h-grown cell suspension to 30 ml of the fresh medium in the incubation vessel. The cells were harvested at desired stages of growth by centrifugation at 4 °C for 10 min at 10000 × *g* and the supernatant was passed through a millipore filter (pore size 0.45 μm). The cells were washed twice in Medium 63, and finally resuspended to a suitable density in Medium 63 which contained chloramphenicol (40 μg/ml). Growth was monitored by the change in absorbance of the culture with a Klett–Summerson colorimeter (filter No. 97).

Chemicals

Thiomethyl-β-D-galactopyranoside (TMG), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and thio-β-D-digalactopyranoside (TDG) were obtained from Mann Research Laboratories. Thio-*o*-nitrophenyl-β-D-galactopyranoside (TONPG) was purchased from Cyclo Chemical Corporation. D-[^{3}H]Fucose was obtained from Amersham Searle. Glycerol was purchased from Fisher Scientific Company; [^{14}]-TMG, [^{3}H]-uracil, L-[U-^{14}C]leucine, L-[^{14}C]proline, and L-[^{14}C]valine were purchased from New England

Nuclear Corporation. Actinomycin D, $NADP^+$, and NADH were purchased from Sigma Chemical Company. Chloramphenicol was a gift from Parke Davis Company. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from Aldrich Chemical.

Assay of intracellular radioactivity

In uptake experiments, a washed cell suspension (usually $A = 400$; 0.9 mg dry wt/ml) was mixed with the radioactive compound under study and incubated at 24 °C (unless otherwise stipulated). At various tine intervals 0.2-ml aliquots were withdrawn and filtered through a millipore filter (0.65 μm pore size) which had been presoaked with Medium 63. Cells were then washed on the filter with 10 ml of Medium 63 and the millipore filter containing the cells was placed in a liquid scinitillation vial. 15 ml of Bray's[6] liquid scintillation fluid was added and the vials were capped, shaken vigorously, and counted in a liquid scintillation counter. During the experiment a 0.1-ml aliquot of cell suspension was placed in 15 ml of Bray's solution and counted. Intracellular concentrations were calculated assuming that 1 ml of cells of absorbance of 100 Klett units (filter No. 42) contained 0.6 μl of intracellular water[7].

Assay for membrane carriers by "Entrance counterflow"

Cells were incubated with 20 mM TMG and 30 mM KN_3 for 30 min at 23 °C. This procedure simultaneously metabolically poisoned the cell and preloaded it with TMG. The suspension was then centrifuged at 10000 × *g* for 10 min, the tube wiped dry with a swab and the cells resuspended in Medium 63 containing [^{14}C]TMG (0.5 mM) and azide (30 mM) at 23 °C. Aliquots (0.2 ml) were withdrawn at various time intervals and filtered through a millipore filter (0.65 μm pore size) which had been presoaked with Medium 63. They were washed and counted as above.

Osmotic shock treatment and spheroplast formation

The methods employed were the same as those previously reported by Heppel[8] except that the sucrose concentration was increased to 45 %. Aliquots were withdrawn from the cell suspension at various intervals, filtered through a millipore filter (0.45 μm pore size), and the filtrates assayed for β-galactosidase and glucose-6-phosphate dehydrogenase activity. Upon the termination of incubation (30 min), the spheroplasts were centrifuged at 30000 × *g*, the pellet washed once in the sucrose medium, and finally lysed in Medium 63. The lysate was assayed for β-galactosidase and glucose-6-phosphate dehydrogenase.

Enzymatic assays

β-Galactosidase was assayed by ONPG hydrolysis. Glucose-6-phosphate dehydrogenase, alkaline phosphatase, and glutamate dehydrogenase were assayed according to the methods of Malamy and Horecker[9].

Labeling of RNA

RNA was labeled by growing the cells in tryptone medium containing 10 μg/ml of [^{3}H]uracil. The cells were harvested at mid-logarithmic phase, washed once with Medium 63, transferred to fresh medium containing 10 μg/ml unlabeled uracil, and allowed to grow until stationary phase was reached. Samples were taken at various intervals, cells separated on millipore filters and an aliquout of the filtrate was

assayed for radioactivity. Phosphorylated and non-phosphorylated products were separated by Dowex anion-exchange chromatography. Yeast RNA was added to the remaining filtrate and precipitated by cold 10 % trichloroacetic acid. The precipitate was collected by millipore filtration, washed with cold 5 % trichloroacetic acid, and radioactivity determined.

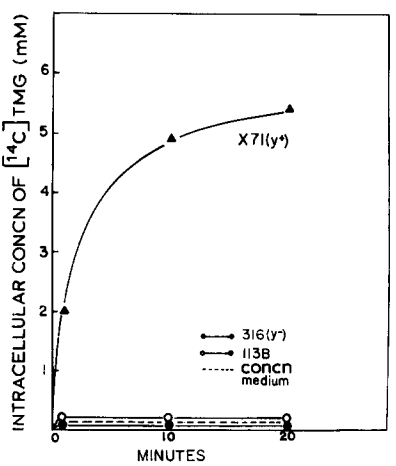
Sephadex chromatography of the culture filtrate

The cells were grown in tryptone medium and harvested in early stationary phase. Following the removal of the cells from the culture medium, the filtrate was concentrated 10-fold by ultrafiltration (UM 10 diaflow ultrafilter, Amicon Corp.). 10 ml of the concentrated solution was put on the Sephadex column and eluted with 0.1 M NaCl at a flow rate of 10 ml/h at 4 °C. 7-ml samples were collected and each read at 280 nm and assayed for β-galactosidase activity. Sephadex G-200 (Pharmacia) was soaked in water for 72 h. It was then packed under gravity in a vertical column 96 cm long, 3.5 cm in diameter, 472-ml volume, and equilibrated with eluant.

RESULTS

Membrane carrier activity in mutants

Several mutants of *E. coli* were isolated which grew on lactose as sole carbon source and fermented this disaccharide on indicator plates, but possessed no membrane transport carriers for lactose. Evidence for the absence of the specific β-galactoside transport system consisted of the following three types of experiments.



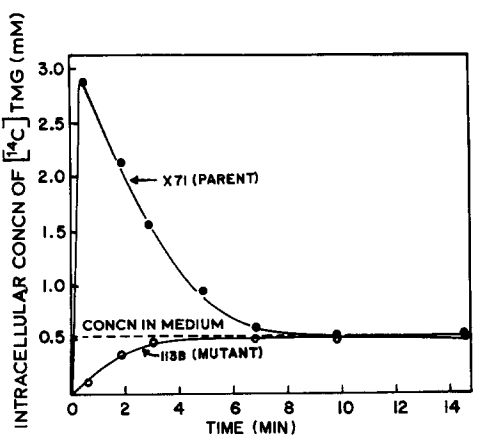


Fig. 1. The accumulation of TMG by X71 and 113B. The washed cells were resuspended in Medium 63 containing 0.025 mM [^{14}C]TMG (0.2 μCi/ml) at an absorbance of 400 KU (filter No. 42) and incubated at 23 °C. Samples were removed at various intervals, millipored, washed and counted.

Fig. 2. TMG counterflow. 4 ml of a cell suspension (A = 200 KU, filter No. 42) were incubated in the presence of 20 mM TMG and 30 mM KN_3 for 30 min at 23 °C. The suspension was then centrifuged, the supernatant decanted and the tube wiped dry. The pellet was resuspended in Medium 63 containing 30 mM KN_3 *plus* 0.5 mM [^{14}C]TMG (0.4 μCi/ml) at 23 °C. Samples (0.5 ml) were removed at various intervals, millipored, washed, and counted.

These mutants were found to lack the capacity to transport TMG against a concentration gradient (see Table I). Fig. 1 shows that 30 s following the exposure of cells of the parental strain (X71) to 0.1 mM TMG the internal concentration was 2.0 mM; under similar conditions mutant 113B showed an internal concentration of 0.04 mM. During the 20-min incubation the internal concentration of TMG in the mutant never exceeded that in the medium. Failure to transport TMG was also found with mutants 241 and 244.

TABLE I

TRANSPORT AND β-GALACTOSIDASE LEAKAGE PROPERTIES OF PARENT AND MUTANTS

Cells were grown in Medium 63 containing 1% tryptone, harvested in early log phase, washed and resuspended in Medium 63. Transport of [^{14}C]TMG was assayed with 0.025 mM substrate. ONPG hydrolysis (1 mM) was measured with a cell density of 2 mg dry wt cells/ml.

Organism	*TMG uptake (% normal)*	*ONPG hydrolysis by intact cells (μmoles ONPG split/min per g dry wt cells)*					*β-Galactosidase in filtrate (μmoles ONPG split/min per l culture filtrate)*
		Control	*formaldehyde +10 mM*	*Inhibition (%)*	*+ TDG 10 mM*	*Inhibition (%)*	
Parent-X71 (y^+)	100	77	12	85	5%	93	0.13
Control-316 (y^-)	0	6	6	0	4	31	0.04
Mutant-241 (y^-)	0	70	68	0	45	35	0.84
Mutant-244 (y^-)	0	65	66	0	45	30	0.72
Mutant-113B (y^-)	0	88	88	0	70	20	5.5
Mutant-415 (15% y^+)	15	125	110	12	90	28	74.0
Mutant-427 (y^+)	100	178	115	35	88	49	7.4

The foregoing data do not unequivocally exclude the presence of membrane transport, however, as energy-uncoupled carriers might be present. Such energy-uncoupled mutants have recently been isolated[10,11] which show a severe defect in TMG accumulation while possessing normal ONPG entry and normal TMG counterflow. Fig. 2 shows that counterflow phenomenon was not found in azide-treated cells of 113B while the typical curve was obtained for similarly treated parental cells.

Additional evidence against the presence of membrane carriers in mutant 113B and other y^- mutants comes from studies of the entry of ONPG into intact cells. Normal carriers are completely inhibited by 10 mM formaldehyde and strongly inhibited by thiodigalactoside (TDG). Table I shows that the three y^- mutants do not possess formaldehyde-sensitive ONPG entry, and the TDG inhibition is very weak. The 20% inhibition by TDG was found to be consistent with a direct inhibition of β-galactosidase, subsequently shown to be in an extracellular location.

Two additional mutants were isolated which appeared to possess both membrane carriers and extracellular β-galactosidase. Mutant 415 showed 15% and mutant 427 showed 100% of normal transport activity as assayed by TMG accumulation (Table I). In addition these two mutants showed large amounts of β-galactosidase in an extracellular position (periplasmic space) as well as leakage into the culture medium.

Periplasmic location of β-galactosidase

Several observations suggest that a significant fraction of the cellular β-galactosidase was located external to the permeability barrier. The first experimental approach was the study of the hydrolysis of ONPG by intact cells of the mutant and parental cells. With normal cells the ONPG enters the cell *via* the lactose transport carrier and is hydrolyzed intracellularly by β-galactosidase to *o*-nitrophenol and galactose. Since transport is rate limiting yellow color production is a good measure of transport activity. Koch[12] has found that 10 mM formaldehyde completely blocked this transport system without affecting β-galactosidase. As shown in Table I formaldehyde inhibited ONPG hydrolysis by the parental strain by 85%. On the other hand the high rate of ONPG hydrolysis by the y⁻ mutants was not inhibited by this agent suggesting that hydrolysis occurs without prior membrane transport. In addition, the transport inhibitor thiodigalactoside (TDG) has relatively less effect on the y⁻ mutants (25 % inhibition) than the parent (93 %). The 25 % inhibition is that due to the direct effect of TDG on β-galactosidase. The rapid ONPG hydrolysis by the mutants could not be reduced by extensive washing of the cells.

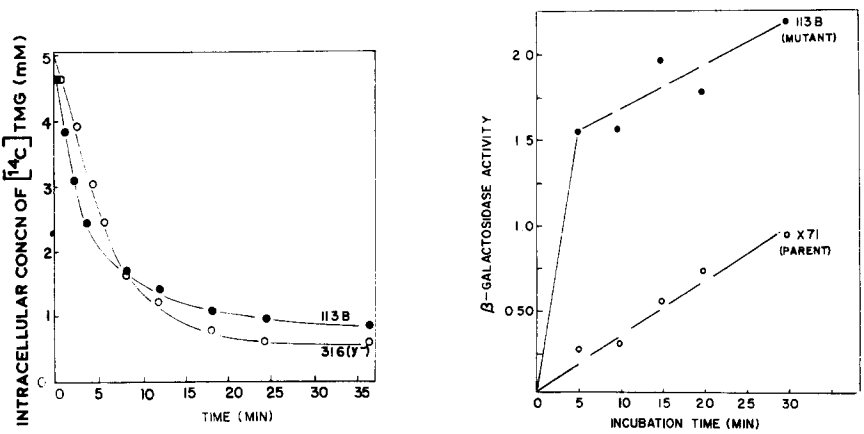


Fig. 3. The efflux of [^{14}C]TMG from 113B and 316 (a transport-negative control which does not leak β-galactosidase). Cells were resuspended at an absorbance of 1400 KU (filter No. 42) in medium containing 5 mM [^{14}C]TMG (12.5 μCi/ml). After incubation for 30 min at 23 °C, the cells were diluted 100-fold into Medium 63 (pH 7.0). 10-ml samples were taken at various intervals, millipored, and radioactivity determined.

Fig. 4. The release of β-galactosidase by X71 and 113B during conversion to spheroplasts. Washed cells were resuspended in a mixture 40 ml of 45% sucrose and 20 ml Tris buffer (0.1 M, pH 8.0) to a final absorbance of 0.60 and placed in ice bath. To this suspension was added 0.05 ml lysozyme (5 mg/ml) and 0.5 ml EDTA (0.1 M). Samples assayed for β-galactosidase activity. Acitivity is expressed in relative units. Spheroplasts began to form immediately and the process was complete in 5 min.

One possible explanation for the formaldehyde-insensitive ONPG hydrolysis was that the mutation affected the plasma membrane in such a manner as to increase its passive permeability to ONPG 10- to 20-fold. According to this hypothesis ONPG would enter the cell through abnormal "leaks" and be split by intracellular β-galactosidase. If such were the case galactosides would be expected to leak out of the cell at an abnormal rate. This was tested experimentally by loading mutant 113B (y⁻) and

316 (y^-) with equal concentration of radioactive TMG, then diluting the cells 100-fold and measuring exit rate of the sugar from the cells. Fig. 3 shows that the rate of "leak" was similar in the two cells. In the case of 427 abnormal "leak" pathways were excluded by the observation that this y^+ organism accumulated TMG to the same high intracellular levels as the parent. Accumulation would be seriously impaired in a cell "leaky" to such sugars. Accumulation of D-fucose and L-leucine were also normal in the mutants tested (Table II). The addition of actinomycin D to the culture medium had no effect on growth of 113B, and NADH was not oxidized when added externally to a cell suspension. Thus, these two agents do not penetrate mutant cells.

TABLE II

D-FUCOSE AND L-LEUCINE ACCUMULATION

The cells were grown in tryptone, harvested in late log phase, washed and resuspended in Medium 63, at an absorbance of 400 KU (filter No. 42), which contained 1.5 μM of D-[^{3}H]fucose or 3 μM of [^{14}C]leucine. Uptake and determination of radioactivity were determined as described in Materials and Methods.

Organism	D-*Fucose* (% *normal*)	L-*Leucine* (% *normal*)
Control X71	100	100
113B	82	100
427	108	89
415	99	84
244	100	97

The most direct evidence that the enzyme is located in the periplasmic space comes from treatment of the cells with lysozyme EDTA. Fig. 4 shows that partial removal of the cell wall of the mutant (113B) caused extensive release of β-galactosidase into the sucrose medium during the first five minutes, while only a very small amount of enzyme is released by the parent. After spheroplast formation was complete (5 min), the rate of release of β-galactosidase occurred at essentially the same rate from 113B and X71. Spheroplast stability was estimated by the rate of release of glucose-6-phosphate dehydrogenase since this enzyme is retained inside the spheroplast, with the result that its release closely paralleled β-galactosidase release from the parent.

A second procedure for the removal of periplasmic protein was the osmotic-shock method of Heppel[8]. Cells were exposed to a sucrose–Tris–EDTA solution and centrifuged. The pellet was resuspended in a hypotonic solution containing $MgCl_2$ and again centrifuged. The "shock fluid" from 113B contained β-galactosidase while that from the parent (X71) contained no detectable enzyme activity.

Release of enzymes into the external medium

At various intervals during growth samples of cell suspension were centrifuged and the supernatant passed through 0.45 millipore filter (to exclude all cells). The cell-free culture medium from mutant cells contained large amounts of β-galactosidase compared with the parental organism. The mutant 113B, for example, spills more than 50 times as much enzyme into the medium when growing on tryptone than does the parent, X71. Fig. 5 shows that the enzyme begins to appear in the culture medium

early in logarithmic phase and continues to increase in concentration through early stationary phase. At the end of the experiment 15–20 % of the total enzyme was found in the medium and 5 % in the periplasmic space. Enzyme also leaks from the cell during growth in glycerol although at a lower level than that observed when cells were grown in tryptone.

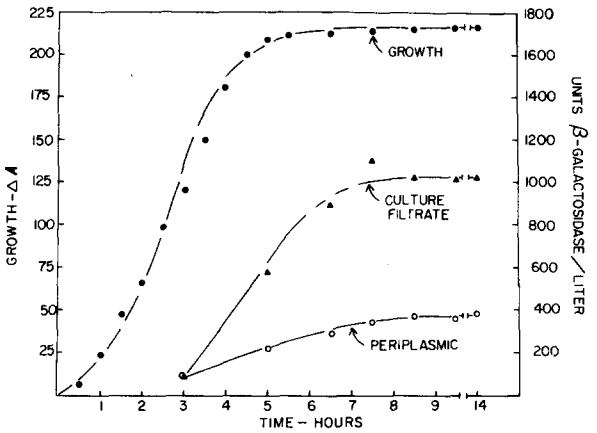


Fig. 5. The correlation between growth and the β-galactosidase activity in a periplasmic location and in the culture filtrate. 1 l of cell suspension was grown in a 2-l flask. Samples were removed periodically through the growth cycle, centrifuged and the supernatant millipored (0.45 μm pore size). Enzyme activity in the culture filtrate is expressed as μmoles of ONPG split/min per l filtrate. Intact cells were washed and exposed to 1 mM ONPG with or without formaldehyde (10 mM). The periplasmic enzyme activity is expressed as μmoles of formaldehyde-insensitive ONPG hydrolysis/min per l of cell suspension.

Other enzymes were also released from the mutant cells during growth. Glucose-6-phosphate dehydrogenase and alkaline phosphatase appeared in the growth medium to a greater extent in cultures of the mutant cell than that of the parent. The total amount of glucose-6-phosphate dehydrogenase lost from the mutant cells at stationary phase was 1 % of that within the cells, with none being lost by the parent. No glutamate dehydrogenase appeared in the culture medium. The mutants (113B, 415, 427) lost all their alkaline phosphatase activity; that is, there was no activity associated with the intact cells or cell free extracts. This is shown in Table III.

What is the specificity of the release of compounds from the cells?

The possibility of the lysis of cells was first considered. Attempts to detect RNA, DNA or free nucleotides in the culture filtrate of 113B were unsuccessful. In an attempt to increase the sensitivity of the method for detecting RNA, it was labeled by growth of 113B in the presence of [^{3}H]uracil. After transfer of cells to fresh medium containing non-radioactive uracil, the release of radioactive products from the cell was measured. No trichloroacetic acid-precipitate counts were found nor was there radioactive phosphorylated material released.

This, *plus* the fact that β-galactosidase release from the cells was gradual and occurred throughout the log phase, was taken as evidence against lysis which usually occurs abruptly. Perhaps the best evidence against lysis is that while considerable

TABLE III

RELEASE OF ALKALINE PHOSPHATASE BY MUTANTS AND PARENT DURING GROWTH

Activity of alkaline phosphatase is determined by the rate of release of *p*-nitrophenol from *p*-nitrophenyl phosphate. Unit enzyme is that liberating one μmole of *p*-nitrophenol per h at 25 °C. The cells were grown to stationary phase in medium containing 0.05 M Tris–HCl buffer (pH 7.5), 10^{-4} *M* sodium glycerol phosphate, 0.2% $(NH_4)_2SO_4$, 0.2% $MgSO_4 \cdot 7H_2O$, $5 \cdot 10^{-5}$% $FeSO_4 \cdot 7H_2O$, $7.5 \cdot 10^{-3}$% KCl, $4 \cdot 10^{-3}$% methionine, 0.5% glucose, 10 μg/ml L-tryptophan, 100 μg/ml L-proline, and 0.5 μg/ml thiamine. Cells were sonicated and diluted to a volume equal to that of the original culture. 0.5 ml of culture filtrate or diluted cell free extract was added to 2 ml of *p*-nitrophenyl phosphate (0.1 mg/ml) in 0.5 M Tris–HCl buffer (pH 8.0), incubated for 1 h, and read at 420 nm on a Beckman DU spectrophotometer.

Organism	*Enzyme activity per ml (μmoles p-nitrophenyl phosphate split per h per ml)*	
	Culture filtrate	*Cell free extract*
X71 (Parent)	17.8	76.8
427	93.7	0
415	71.0	0
113B	80.6	0

amounts of β-galactosidase were found in the medium very little glucose-6-phosphate dehydrogenase and no glutamate dehydrogenase were found.

Additional experiments were performed to determine whether proteins other than β-galactosidase were lost from the mutant cells. Cells of 113B (mutant) and X71 (parent) were grown to late log phase in Medium 63 containing tryptone as carbon source. Cells were centrifuged and the supernatant passed through millipore filters (pore size 0.45), concentrated 10-fold by ultrafiltration, and chromatographed on Sephadex G-200. The elution profiles obtained with 113B and X71 are shown in Fig. 6. While peaks A and B are present in the culture filtrate of the parent, the amount in

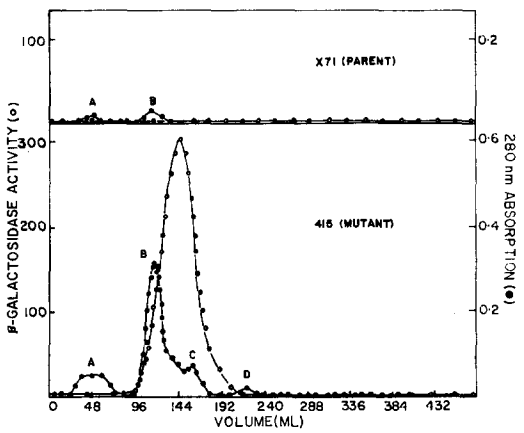


Fig. 6. Sephadex G-200 chromatography of culture medium. The cells were removed by centrifugation followed by millipore filtration of the supernatant. The culture filtrate was concentrated 10-fold by ultrafiltration and chromatographed on a column of Sephadex G-200. ●—●, absorbance at 280 nm; ○—○, β-galactosidase activity. Enzyme activity is expressed in relative units.

the culture filtrate of the mutant was increased many fold. β-Galactosidase and Peaks C and D were not evident in the culture filtrate of the parent.

Temperature sensitivity

When each of the five mutants were grown at four different temperatures (23, 30, 37, and 42 °C) it was observed that the leakage of β-galactosidase was extensive at all temperatures, although slightly greater at the higher temperatures. The transport capacity of the cells, as measured by ONPG hydrolysis and TMG uptake, was unaltered by variation in the growth temperature.

Osmotic fragility

Varying the osmolarity of the incubation medium had a profound effect on the mutant cells. Growth of 113B in tryptone *plus* 20 % sucrose reduced the leakage of β-galactosidase to 30 % of the levels observed in the absence of sucrose. Exposure of mutant cells to distilled water for 30 min reduced their viability to 10 % of the level found prior to this treatment. The viability of the parental cells was unaffected by this procedure.

DISCUSSION

Leakage of β-galactosidase from *E. coli* may result from alterations of the plasma membrane–cell wall complex due to external agents such as chemicals or irradiation[13] or due to a variety of cellular abnormalities as a consequence of mutation. Ricard *et al.*[14] have reported several mutants of *E. coli* K-12 which leak this enzyme. These lactose positive mutants showed extremely rapid ONPG hydrolysis by intact cells which was attributed to an abnormally rapid entry rate of the sugar. An alternative explanation, namely the periplasmic location of some of the β-galactosidase (as found in the present study) was apparently not considered. More recently, Crandall and Koch[15] have isolated several temperature-sensitive mutants of *E. coli* ML-308 some of which leak β-galactosidase, loose β-galactoside transport, and show some other defects at the non-permissive temperature (42 °C). Lopes *et al.*[16] have isolated mutants of *E. coli* which leak periplasmic enzymes (RNAase, cyclic phosphodiesterase, and 5′-nucleotidase) but do not release cytoplasmic enzymes. Growth was also impaired in some of those mutants at 42 °C. They concluded that leakage was due to a defect in the outer cell membrane of the cell.

The mutants described in this paper appear to possess a defect in the plasma membrane–cell wall complex since leakage of several proteins occurs first into the periplasmic space and then into the extracellular medium. Furthermore the mutants are more fragile to hypotonic medium than the parental cells. Exposure of mutants to distilled water causes loss of viability and addition of 20 % sucrose to the growth medium reduces enzyme leakage. Both of these observations suggest an abnormality in the rigid portion of the cell wall. There appears to be no relationship between the lactose-transport system and the cell membrane–cell wall defect in these mutants. Further studies on the possible enzymatic defect in the mutants are in progress.

Special note should be made of the physiological consequences of this particular defect. Extracellular hydrolysis of large molecules followed by uptake of the low-molecular-weight products is frequently encountered in both animal and bacterial

cells. *E. coli*, for example, utilizes its periplasmic enzymes such as phosphatases for exactly this purpose. β-Galactosidase which is normally entirely intracellular in *E. coli* is present in a periplasmic and extracellular position in the mutants described in this paper. Under these conditions lactose is hydrolyzed external to the plasma membrane and the split products glucose and galactose are subsequently metabolized. Thus growth of these cells in lactose occurs in the absence of the lactose-transport system. The selective advantage conferred on the cell with a membrane transport system and intracellular hydrolytic enzymes is probably its ability to scavenge very low concentrations of lactose and to effectively prevent loss of the split products.

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